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MONOCLONAL ANTIBODY INHIBITOR OF GM-CSF, IL-3, IL-5 AND OTHER CYTOKINES, AND USES THEREOF

FIELD OF THE INVENTION

5   This invention relates to a method of isolating monoclonal antibody inhibitors and
reagents derived therefrom and other inhibitors of cytokine binding including
monoclonal antibodies and reagents derived therefrom and small molecules capable
of inhibiting binding of GM-CSF, IL-3 and IL-5 to the common beta receptor
subunit.

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INTRODUCTION

Human interleukin (IL)-5, IL-3 and granulocyte-macrophage colony-stimulating
factor (GM-CSF) are cytokines involved in hemopoiesis and inflammation
(Metcalf; 1986). All three cytokines stimulate eosinophil production, function and
15   survival (Metcalf; 1986) and therefore have the ability to influence inflammatory
diseases such as asthma, atopic dermatitis and allergic rhinitis where the eosinophil
plays a major effector role. IL-5, being the eosinophil specific cytokine, has
received most of the initial attention with IL-5 mRNA and protein levels noted to
be elevated in lung tissue and bronchoalveolar lavage (BAL) fluid from
20   symptomatic asthma patients (Fukuda et al 1994). Correlation between IL-5 levels
and allergen challenge and disease activity have also been seen (Sur et al, 1996).
It is becoming apparent, however, that not only IL-5 but also GM-CSF and IL-3
play a role in eosinophil production and activation in asthma as there is evidence of
both GM-CSF and IL-3 being synthesized at sites of allergic inflammation (Bagley
25   et al, 1997b; Allen et al 1997). It is possible that expression of these cytokines
contributes to the total number of infiltrating eosinophils and the degree of
eosinophil activation. Alternatively, they may be responsible for different phases
of eosinophil infiltration. Recent kinetic data from patients undergoing antigen
challenge showed that IL-5 levels increased between days 2-7 post challenge,
30   whilst GM-CSF peaked at day 2, and remained elevated throughout day 16.
Furthermore, GM-CSF detection extended beyond the site of allergen challenge.

IL-5, GM-CSF and IL-3 stimulate eosinophils and other normal and cancer cells
by binding to cell surface receptors that comprise a ligand-specific α chain and a β
35   chain which is shared by the three receptors (βc) (Bagley et al 1997a). Binding to
each receptor α chain is the initial step in receptor activation, however, engagement
of either α chain alone is not sufficient for activation to occur. Recruitment of βc

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by each ligand: α chain complex follows, a step that has two major functional consequences: firstly, it allows the binding of IL-5, GM-CSF and IL-3 to become essentially irreversible; and secondly, it leads to full receptor activation (Bagley *et al* 1997a). Since β_c is the major signalling component of these receptors its

5   engagement leads to the activation of JAK-2, STAT-5 and other signalling molecules culminating in the full plethora of cellular activities commonly associated with either IL-5, GM-CSF and IL-3 stimulation such as eosinophil adherence, priming for degranulation and cytotoxicity, and prolongation of viability (Bates *et al*, 1996).

10

In order to block or antagonize the activity of eosinophil-activating cytokines *in vivo* three major approaches are being tried. One of them utilizes antibodies to the implicated cytokines. For example, antibodies to IL-5 are being used in an animal model of allergen-induced asthma and have shown to have a relatively long lasting

15   effect in preventing eosinophil influx into the airways and bronchial hyperresponsiveness (Mauser *et al*, 1995). A second approach relies on IL-5 or GM-CSF mutants which can bind to the respective α chains with wild type affinity but which have lost or shown reduced ability to interact with human β_c. IL-5 mutants such as E13Q, E13K and E13R, and the human GM-CSF mutant E21R

20   directly antagonize the functional activation of eosinophils by IL-5 or GM-CSF respectively (Tavernier *et al* 1995; McKinnon *et al* 1997; Hercus *et al* 1994b). However, at least in the case of E13K, eosinophil survival is not antagonized and in fact this mutant is able to support eosinophil survival (McKinnon *et al* 1997). A third approach involves the use of soluble receptor α chains which can sequester

25   circulating cytokines. However, this carries the risk of a cytokine:receptor α chain complex potentially interacting with surface-expressed β_c and triggering receptor activation. The common theme amongst these approaches is that they tackle a single receptor system involving either IL-5, GM-CSF or IL-3 leaving the other two eosinophil-acting cytokines unaffected. Although the concomitant

30   administration of IL-5 and GM-CSF antagonists may be considered, this may be clinically impracticable.

An alternative approach to blocking eosinophil-activating cytokines involves targeting the common β chain of their receptors. Although β_c does not directly

35   bind IL-5, GM-CSF or IL-3 alone, it does bind to these cytokines complexed to the appropriate receptor α chain. Lopez *et al* in WO 97/28190, which is incorporated herein by reference in its entirety, have identified the major binding

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sites of, βc for the IL-5:IL-5Rα, GM- CSF:GM-CSFRα and IL-3:IL-3Rα
complexes. Significantly, these sites are utilized by all three complexes and
comprise the predicted B'-C' loop and F-G' loop in βc. Thus targeting βc is not
on y desirable but also feasible, with the added potential to allow the simultaneous
5   inhibition of IL-5, GM-CSF and IL-3 action by a single agent. These workers
have shown that certain mutants in the B'-C' and the F-G' loop fail to bind IL-5,
GM-CSF and IL-3.

SUMMARY OF THE INVENTION
10 The present invention results from the isolation of a monoclonal antibody (BION-
1) raised against the membrane proximal domain (domain 4) of βc which is able to
block the production and activation of human eosinophils stimulated by IL-5,
GM-CSF or IL-3 and blocks the growth of leukaemic cell lines. This MoAb was
able to block the high affinity binding of all three cytokines to eosinophils by
15 binding to residues in the predicted B'-C' and F-G' loops of βc, and prevented
receptor dimerization and βc phosphorylation. It was found that raising an
antibody capable of blocking the binding of all three cytokines was possible by
screening monoclonal antibody-expressing hybridoma cell lines arising from
immunising mice with cells expressing only domain 4 of β_c and lacking domains 1
20 to 3 and expressing domain 4 and the transmembrane and cytoplasmic regions.

Additionally this finding is likely to have implications for other members of the
cytokine receptor superfamily some of which are shared subunits in a given
subfamily (that is they bind several cytokines), and some which are ligand specific
25 and bind to only one cytokine. The receptor α-chains for GM-CSF, IL-3 and IL-5
and β_c belong to the rapidly expanding cytokine receptor superfamily. Within this
superfamily several sub-families are now emerging that are characterized by the
sharing of a communal receptor subunit by multiple ligands: gp130 acts as an
affinity converter and signal transducer for IL-6 (Hibi et al., 1990; Taga et al.,
30 1992), IL-11 (Hilton et al., 1994), oncostatin M (Liu et al., 1992), ciliary
neurotrophic factor, leukaemia inhibitory factor (LIF) (Ip et al., 1992) and
cardiotrophin-1 (Pennica et al., 1995); the LIF receptor (LIFR) also binds ciliary
neurotrophic factor (Davis et al., 1993), cardiotrophin-1 (Pennica et al., 1995) and
oncostatin M in addition to LIF (Gearing et al., 1994); IL-2R β supports affinity
35 conversion and signalling of IL-2 and IL-15 (Giri et al., 1994); IL-2R γ chain
affinity converts IL-2 (Takeshita et al., 1992), IL-4 (Russell et al., 1993), IL-7
(Noguchi et al., 1993), IL-9 (Kimura et al., 1995) and IL-15 (Giri et al., 1994);

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evidence also suggests that IL-4 and IL-13 share a receptor component (Zurawski et al, 1993) and this subunit has recently been cloned (Hilton et al., 1996). It is not known which residues in gp130, LIFR and IL-2R β and γ chains are important for ligand binding or indeed whether different ligands share or have unique sets of

5   binding determinants on these communal receptor subunits. Because these common subunits are vital for transducing signals by several ligands, the possibility arises that interfering with the ability of these common subunits to bind ligand or to form homodimers may affect the action of more than one ligand.

10 Clear similarities in structure between β_c and other cytokine receptors have been recognised and similarities in at least part of the binding site, the F-G' loop, have been identified in Lopez et al in WO 97/28190. Accordingly it is an expectation that the means employed by the inventors to obtain a monoclonal antibody that inhibits binding of IL-3, GM-CSF and IL-5 will also lead to the isolation of

15 monoclonal antibodies that inhibit binding of other cytokines to their respective receptors.

In a broad form of a first aspect the invention could be said to reside in a method of isolating a monoclonal antibody capable of inhibiting any one of IL-3, GM-CSF

20 and IL-5 binding to the common receptor β_c, or a monoclonal antibody capable of inhibiting a cytokines binding to a receptor analogous to β_c, said method comprising the step of immunising an animal with a cytokine receptor or portion of a cytokine receptor containing the critical binding site which portion might include the extracellular domain 4 or analogous domain in the analogous common receptor

25 or part thereof, isolating antibody producing cells from said animal and fusing antibody producing cells with a myeloma cell line, screening for a cell line that produces an antibody of the desired type.

The immunisation may involve introducing a cDNA clone of a portion of or all of

30 the common receptor including the extracellular domain 4 or analogous domain in the analogous common receptor or part thereof, into a cell and proliferating said cells to form a recombinant cell line, inoculating an animal with said recombinant cell line, isolating antibody producing cells from said animal and fusing the antibody producing cell line with a myeloma cell line to form a hybridoma cell line,

35 screening for a hybridoma cell line that produces an antibody that binds to the recombinant cell line but not to the parent, and then testing for inhibition against all

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three cytokines. In one form the cell into which the cDNA clone is introduced is
mammalian and one commonly used mammalian cell line is a COS cell.

The cDNA may encode a full or partial portion of domain 4 when it is in a
5   configuration where the F'-G' loop and/or the B'-C' loop is in its native shape.
The data below show that cDNA encoding substantially only domain 4 of the
extracellular portion of β_c as well as the transmembrane and the intracellular
portions maintains these sites in a sufficiently integral conformation so that an
antibody raised thereagainst will give the inhibition sought. It is postulated that the
10 same will be the case for analogous receptors for the cytokine superfamily. This
method should be distinguished from immunising with the whole receptor since
the extracellular domain 4 is likely to be covered or masked by other domains in
the whole receptor.

15 β_c has two repeats of the cytokine receptor module (CRM), each of which has two
discrete folding domains (CRDs), so that in total β_c has 4 domains hence named
domains 1 to 4 ($\beta 1$ to 4). It is postulated that domain 2 of any CRM may be an
equivalent of domain 4 and therefore domain 2 may be used in the immunisation.

20 In addition the domain 4 of β_c or equivalent domain in other cytokine receptors
may be expressed in isolation in a microbial host such as *Escherichia coli* and used
to immunise animals for developing monoclonal antibodies.

The analogous receptor may be any one of the cytokine superfamily receptors but
25 not limited to the group comprising β_c, LIFR, gp130, IL-2Rβ, IL-4R/IL-13R, IL-
2Rγ, IL-3Rα, EPOR, TPOR and OBR.

It will be understood that in one specific form of this aspect of the invention the
method is used to isolate a monoclonal antibody that inhibits cytokine binding to a
30 common receptor subunit. The common receptor is envisaged to be selected from
the group of receptors acting for more than one cytokine including but not limited
to gp130, LIFR, IL2Rβ/IL2Rα, and IL-4R/IL-13R in addition to β_c.

It will also be understood that the invention encompasses monoclonal antibodies or
35 fragments thereof produced as a result of this first form of the invention.

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→ In a broad form of a second aspect the invention could be said to reside in a monoclonal antibody, or fragments thereof capable of inhibiting the binding of the three cytokines IL-3, GM-CSF and IL-5 to the β_c receptor.

5 The degree of inhibition may range from complete inhibition to moderate inhibition, which inhibition will of course be dependent on the amount of monoclonal antibody or fragments thereof added to inhibit and the relative affinity of the antibody or fragment thereof to the β_c.

10 The extent of inhibition of respective ones of the three cytokines is not necessarily identical and may vary, so the different cytokines may be inhibited from binding to different degrees.

The antibody fragments may be larger portions such as Fab fragments or much
15 smaller fragments of the variable region. These fragments may be used as separate molecules or alternatively may form part of a recombinant molecule which is then used for therapeutic purposes. Thus for example the monoclonal antibody may be "humanised" by recombining nucleic acid encoding the variable region of the monoclonal antibody with nucleic acid encoding non-variable regions of human
20 origin in an appropriate expression vector.

The inhibition preferably leads to blocking of at least one function of all three cytokines. One of the benefits that is proposed to be derived from these antibodies or antibody fragments is their use in modifying cells stimulated by one of the three
25 cytokines, and more in one specific form modifying the activity of the three cytokines is proposed to impact greatly on eosinophil function. Therefore preferably the activity leads to inhibition of stimulation of effector cell activation and where the antibody or fragment thereof is to be used for treatment of asthma leads most preferably to inhibition of IL-5, IL-3 & GM-CSF mediated eosinophil
30 activation. It will be understood however that cells other than eosinophils are also the effectors of adverse conditions in humans and animals as a result of stimulation by these cytokines and inhibition of such stimulation is also contemplated by this invention. These include cells that express either one or all of GM-CSF, IL-3 and IL-5 receptors, the stimulation of which leads to pathology. Examples of these are
35 leukaemic cells, endothelial cells, breast cancer cells, prostate cancer cells, small cell lung carcinoma cells, colon cancer cells, macrophages in chronic inflammation

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such as rheumatoid arthritis, dendritic cells for immunosuppression and neutrophils in inflammation.

5 This in one form the invention may be said to reside in an inhibitor of leukaemic cell growth wherein the inhibitor is capable of inhibiting the binding of one or all of IL-3, GM-CSF and IL-5 to the β_c receptor. The inhibitor may be BION-1 or an agent capable of inhibiting BION-1 binding with β_c.

10 A number of different facets of eosinophil function might be modified so that in one form IL-5, IL-3 & GM-CSF mediated eosinophil survival is inhibited or blocked. In a second form IL-5, IL-3 and GM-CSF mediated eosinophil activation is inhibited or blocked.

15 In one form of this second aspect of the invention the monoclonal antibody or fragment thereof binds to at least the F'-G' loop of domain 4 of the β_c subunit.

In an alternative form the monoclonal antibody or fragment thereof binds to at least the B'-C' loop of domain 4 of the β_c subunit but this alternative form is not limited to monoclonal antibodies or fragments thereof that only bind to the F'-G' loop but
20 includes monoclonal antibodies or fragments thereof that perhaps binds to both the F'-G' as well as the B'-C' loop of domain 4 of the β_c.

It is thought that the monoclonal antibody isolated by the inventors inhibits dimerisation of the common receptor units and thus the invention might encompass
25 an antibody or fragments thereof of the second aspect of the invention that inhibit β_c receptor dimerisation.

In one very specific form the monoclonal antibody is the antibody produced by the hybridoma cell line BION-1 (ATCC HB-12525).
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In a broad form of a third aspect the invention could be said to reside in a hybridoma cell line capable of producing a monoclonal antibody of any form of the first or second aspect of the invention.

35 In one specific form of the third aspect of the invention the hybridoma cell line is BION-1 (ATCC HB-12525).

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Since GM-CSF, IL-3 and IL-5 need to bind their respective α chains before being able to interact with β_c, at present most screening for new inhibitors utilise cell-based assays where both, α and β_c receptor units are co-expressed. Solid phase assays rely on inhibition of GM-CSF, IL-3 or IL-5 to their respective α chain only

5   since these cytokines cannot bind to β_c alone. Since BION-1, unlike these three cytokines, can directly bind to β_c we propose that it can be used as a novel solid phase screening assay. Any compound that binds the appropriate site which is likely to inhibit all three cytokines will also inhibit the binding of BION-1. Additionally once further inhibitory compounds are uncovered these could be used

10   in the place of BION-1 in that screening process. This therefore facilitates the screening of larger number of candidate inhibitor compounds.

In a broad form of a fourth aspect therefore the invention could be said to reside in a method of screening peptides, oligonucleotides and other small molecules for

15   their capacity to competitively inhibit the binding of BION-1 or the binding of an agent capable of inhibiting BION-1 binding, to the β_c subunit.

Generally the screening assay involves contacting BION-1 or fragment thereof with the β_c subunit or fragment thereof as well as a candidate inhibitory

20   compound, and measuring the degree of binding.

A reporting means is preferably provided to facilitate the detection of binding of BION1 or fragment thereof with β_c subunit or fragment thereof. Thus, for example, a competitive binding assay using labelled BION-1 could be used for this

25   purpose. β_c or domain 4 of β_c is immobilized on a plate or tube and several compounds added, followed by labelled or tagged BION-1 or fragments thereof. Since BION-1 binds the region of β_c involved in binding all three cytokines, any compounds that block or reduce the binding of BION-1 or fragments thereof to β_c or domain 4 will be considered candidate inhibitory compounds. Thus, the

30   availability of BION-1 as an agent that for the first time allows the direct binding to the cytokine binding region of β_c affords a novel test for the identification of simultaneous inhibitors of GM-CSF, IL-3 and IL-5. It will be understood that the same will apply for other cytokines and their respective receptors.

35   It will be understood that not the entire β_c subunit needs be used to screen candidate compounds, and certainly the present data indicates that a fragment of the

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β_c subunit encompassing domain 4 has sufficient structure in common with the native β_c subunit to reflect the configuration of the cellular target for an inhibitor useful for an *in vivo* effect.

5   In a broad form of a fifth aspect, the invention could be said to reside in a cytokine inhibitor capable of simultaneously blocking the binding of β_c by IL-3, GM-CSF, and IL-5 made according to the fourth aspect of the invention.

It is thought that compounds that inhibit binding of the IL-3, IL-5 and GM-CSF to
10   the β_c will be therapeutically useful for intervention in conditions where IL-3, GM-CSF and IL-5 play a pathogenic role, mainly allergy, asthma, acute and chronic myeloid leukaemias, lymphoma and inflammation including rheumatoid arthritis, breast cancer and prostate cancer.

15   Similarly for other common cytokine receptors it is thought that antagonists or agonists will be therapeutically useful. gp130 is functionally analogous to β_c in that it is a common binding sub-unit and signal transducer for the IL-6, oncostatin M (OSM), ciliary neutrotrophic factor (CNTF), leukaemia inhibitory factor (LIF) and IL-11. It is suggested that raising an antibody against a domain analogous to
20   domain 4 of β_c will also lead to blocking of two or more of these cytokines. Antagonism of this receptor system will be useful in inflammation, leukaemia and lymphoma. Antagonists to IL2Rβ/IL2Rα may be useful as immunosuppresants. Antagonists of LIFR may be useful for the prevention of implantation of embryos in uteri. Antagonists of IL-4/IL-13 will inhibit IgE production and may be useful
25   in treating asthma and allergies.

BRIEF DESCRIPTION OF THE DRAWINGS

       Figure 1.   Flow cytometry analysis of the staining of MoAb BION-1
30   (continuous line) and an isotype matched IgG$_1$ control MoAb
                     (dotted line) to (A) COS cells transiently transfected with β_c,
                     (B) CHO cells constitutively expressing β_c, (C) TF-1.8 cells,
                     (D) neutrophils, (E) eosinophils and (F) monocytes.

35   Figure 2.   MoAb BION-1 recognizes β_c protein. (A)
                     Immunoprecipitation of β_c from ^{125}I-surface labelled CHO β_c

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cell lysate. Both experiments were performed on 7.5% SDS
PAGE under reducing conditions. (C) MoAb BION-1, but
not another anti-βc MoAb 1C1, recognises a βc mutant
(βc-ΔQP) that contains only domain 4 in the extracellular
5   regions by immunoprecipitation of CHO ΔQP cells. This
mutant has a flag attached so it can also be seen by anti-flag
MoAb M2. The experiment was performed on 10% SDS
PAGE under reducing conditions.

10   Figure 3.   Dose-dependent competition for the binding of ^{125}I-IL-5 (50
pM), ^{125}I-GM-CSF (50 pM) and ^{125}I-IL-3 (200 pM) by
MoAbs BION-1 (•), an anti-βc MoAb control ( ), and an
IgG$_1$ control MoAb (o) to TF-1.8 cells (2×10^6 per point). (---
) represents ligand binding in the presence of 200-fold excess
15   unlabelled ligand. Each point is the mean of triplicate
determinations.

    Figure 4.   A Fab fragment of BION-1 blocks high affinity binding of
IL-5, GM-CSF and IL-3. Binding of ^{125}I-IL-5 (50 pM),
20 ^{125}I-GM-CSF (50 pM) and ^{125}I-IL-3 (200 pM) were assessed
on TFI.8 cells (2×10^6 per point) in the presence or absence of
3000 nM MoAb BION-1, or control anti-βc MoAb, or 4200
nM Fab fragment of BION-1 for 2 h at room temperature.
1C1 was used as a non blocking anti-βc control. Cells were
25   separated from unbound radioligand by spinning through
FCS and the resulting cell pellet was counted. The results are
expressed as a percentage of the total specific cpm bound seen
in the absence of antibody. Non-specific binding was
determined in the presence of 200 fold excess of cold ligand.
30   Total binding seen for ^{125}I-IL-5, ^{125}I-GM-CSF and ^{125}I-IL-3
were 9744.2, 2567.1 and 3379.13 cpm respectively.
Blocking with Fab fragment of BION-1 was determined from
a single point and BION-1 and 1C1 values are the mean of
duplicate determinations with error bars representing 1
35   standard deviation.

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Figure 5.   BION-1 blocks high affinity binding of IL-5, GM-CSF and IL-3 to human eosinophils. Human eosinophils (1.8×10^6 per point) were incubated with ^{125}I-IL-5 (10 pM or 3 pM), ^{125}I-GM-CSF (50 pM) or ^{125}I-IL-3 (200 pM) either alone or in the presence of 1μM MoAb at room temperature for 2 h. MoAb's 9E10 and 8E4 were used as isotype matched and non-blocking anti-βc control respectively for BION-1. Cells were separated from unbound radioligand by spinning through FCS and the resulting cell pellet was counted. The results are expressed as a percentage of the total specific cpm bound seen in the absence of antibody. Non-specific binding was determined in the presence of 200 fold excess of cold ligand and was determined to be an average of 0.3% of total counts added. Total binding seen for ^{125}I-IL-5, ^{125}I-GM-CSF and ^{125}I-IL-3 were 765, 622 and 748 cpm respectively. Each point is the mean of duplicate determinations and error bars represent 1 standard deviation.

Figure 6.   MoAb BION-1 recognizes an epitope in βc comprising at least residues M363, R364, E366 and R418. Human βc wild type and mutants (Woodcock et al, 1996) were tested for reactivity with MoAb BION-1 and 1C1 used as a control. Following expression of wild type βc and βc mutants on COS cells, βc was immunoprecipitated by MoAb 8E4, followed by Western blotting by MoAb BION-1 (top) or the non blocking MoAb 1C1 (bottom).

Figure 7.   The binding of ^{125}I-labelled MoAb BION-1 (1 nM) to TF.1 cells is inhibited by IL-3 (•), but not by TNF-α (o). (---) Represents inhibition in the presence of 200-fold excess of unlabelled BION-1.

Figure 8.   BION-1 IgG selectively inhibits IL-5, GM-CSF and IL-3 mediated proliferation of TF 1.8 cells. The proliferation experiments represent the comparison of inhibition of BION-1 IgG at maximal dosage (400 μg/ml BION-1 with IL-5 and IL-3 and 850 μg/ml with GM-CSF) against ED^{50}

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concentrations for IL-5 (0.3 ng/ml), GM-CSF (0.03 ng/ml)
or IL-3 (0.3 ng/ml). An anti-βc antibody and an irrelevant
IgG antibody were used as controls. The results are
expressed as DPM. Each value represents the mean of
5   triplicate determinations and error bars represent the SEM.

Figure 9.   Fab fragment of BION-1 and BION-1 IgG inhibits IL-5,
                 GM-CSF and IL-3 mediated proliferation of TF1.8 cells.
                 Intact IgG or Fab fragment of BION-1 were titrated against a
10   fixed concentration of IL-5 (0.3 ng/ml), GM-CSF (0.03
                 ng/ml) or IL- 3 (0.3 ng/ml) in proliferation assays where
                 TF1.8 cells at 5×10^4/ well were incubated for 48 hours and
                 then pulsed for 5 hours with 0.5 μCi/well ^{3}H-Thymidine.
                 The results are expressed as DPM. Each value represents the
15   mean of triplicate determinations and error bars represent the
                 SEM.

Figure 10.   Eosinophil survival. (A) Viability of eosinophil after 36
                 hours in the presence of IL-5, IL-3 and GM-CSF. (B)
20   Viability of eosinophil after 36 hours in the presence of IL-5,
                 IL-3 and GM-CSF (1 nM) and different concentrations of
                 MoAb BION-1 (o) and 8E4 (•). Each point is the mean of
                 triplicate determinations from three samples and error bars
                 represent 1 standard deviation.

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Figure 11.   MoAb BION-1 inhibits IL-5-stimulated CD69 up-regulation
                 on human eosinophils. (A) CD69 up-regulation in the
                 presence of different concentrations of IL- 5, IL-3, GM-CSF
                 and TNF-α. (B) CD69 up-regulation stimulated by 1 nM of
30   IL-5, GM- CSF, IL-3 or TNF-α in the presence of different
                 concentrations of MoAb BION-1 or control anti-βc MoAb
                 8E4. Each point is the mean value of three replicates and
                 error bars represent 1 standard deviation.

35   Figure 12. Inhibition of IL-3-induced α and β chain dimerization and
                 phosphorylation by MoAb BION-1. Immunoprecipitations
                 using anti-IL-3Rα MoAb 9F5 or anti-βc MoAb 8E4 from

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M07e cells preincubated with MoAbs BION-1, MoAb 1C1 or medium alone (-) for 1 min, before being stimulated (+) or not (-) with IL-3 (50 nM) for 5 min. The figure was visualised by PhosphorImaging and the position and
5   molecular weight (in thousands) of marker proteins are shown to the left of the gels. The gels were reprobed by Western blotting analysis using anti-phosphotyrosine MoAb 3-365-10 and the top panel shows the image of part of the gels in the βc area.

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Figure 13.   Screening peptides for inhibition of MoAb BION-1 binding to soluble β_c domain 4 adsorbed to solid phase. *E. coli* derived soluble β_c domain 4 ($s\beta_c$#4) was coupled to Maxisorp ELISA plates at 10μg/ml in 0.1M carbonate buffer overnight and then
15   blocked with 1% BSA. (A) B45.pep (FHWWWQP-GGGCDYDDDK) (÷) and (B) YB12.pep (FPFWYHAHSPWS-GGGCDYKDDDK) (•) were derived from biopanning libraries with $s\beta_c$#4 using an acid eluant. B45 was allowed the bond to $s\beta_c$#4 at 0.0125μM and YB12
20   was allowed to bind $s\beta_c$#4 at 0.025μM. BION-1 was added to the plates at a starting concentration of 5μg/ml and serial dilutions were used to titrate the BION-1 down to 0.004μg/ml. The plate was washed again and BION-1 binding to $s\beta_c$#4 was detected.

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Figure 14.   BION-1 specifically inhibits the growth *in vitro* of chronic myelomonoctic cells (CMML). A contol antibody (1C1) does not inhibit.

30   DETAILED DESCRIPTION OF THE INVENTION .

Mat.rials and Methods

Δ*QP cDNA:* To express domain 4 of βc on the cell surface we cloned the activated
35   βc mutant, hβcΔQP, with an extracellular deletion removing domains 1 to 3

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(D'Andrea *et al* 1996), into the eukaryotic expression vector pcDNA3
(Invitrogen).

Cytokines and cell lines: Recombinant human IL-3 and GM-CSF were produced
5   in *E. coli* as described (Barry *et al* 1994, Hercus *et al* 1994b). Recombinant human
IL-5 was purified from *E.coli* by Bresatec (Adelaide, South Australia).
Recombinant EPO was purchased from Johnson & Johnson (New Jersey).
TNFα was a gift from Dr. J. Gamble in the Hanson Centre for Cancer Research.
COS cells were transfected with receptor cDNA as described previously
10   (Woodcock *et al* 1994). CHOβc and CHOΔQP cells stably expressing either full
length βc or domain 4 respectively were generated by electroporation (Hercus *et al*,
1994a). TF1.8 cells were a gift from Dr J. Tavernier from University of Gent,
Belgium. MO7e cells, a human megakaryoblastic cell line, were from Dr P
Crozier, Aukland, New Zealand. Human eosinophils were purified from the
15   peripheral blood of slightly eosinophilic volunteers via sedimentation through
dextran and centrifugation through a discontinuous density gradient of hypertonic
Metrizamide, as previously described (Vadas *et al* 1979). Eosinophils were more
than 92% pure. Human neutrophils and monocytes were purified from peripheral
blood as described previously (Lopez *et al*, 1990) with more than 95% purity.
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Generation of anti-βc MoAbs: BALB/c mice were immunized intraperitonally with
1×10^7 COS cells transfected with βc or ΔQP expression constructs. ΔQP
constructs express substantially only domain 4 of the extracellular domains of βc.
The immunizations were repeated 4 times at two-weekly intervals. Four weeks
25   after the final immunization, a mouse was boosted with 2×10^6 COS transfectants
intravenously. Three days later, splenocytes were harvested and fused with NS-1
myeloma cells as previously described (Sun *et al* 1996). Hybridoma supernatants
were screened on CHO βc or CHO ΔQP cells by flow cytometry, with
untransfected CHO cells as a control. All antibodies were from single hybridoma
30   clones as selected by limiting dilution method. MoAbs were purified from ascites
fluid or hybridoma supernatant by a protein A sepharose column. The isotypes of
MoAbs were tested with a Mouse MoAb Isotyping Kit (Boehringer Mannheim,
Germany). Fab fragments were generated using a Fab Preparation kit (Pierce,
Rocford, IL) following the supplied protocol.
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Immunofluorescence: Freshly purified neutrophils, eosinophils, monocytes, or CHO and COS cell transfectants (5×10^5) were incubated with 50μl of hybridoma supernatant or 0.25mg of purified MoAb for 45-60 min at 4°C. Cells were washed twice and then incubated with FITC-conjugated rabbit anti-mouse Ig
5   (Silenus, Hawthorn, Victoria, Australia) for another 30-45 min. Cells were then washed and fixed before analysing their fluorescence intensity on an EPICS-Profile II Flow Cytometer (Courter Electronics). Two colour staining was carried out by additional incubation with another MoAb directly coupled to PK.

10   *Ligand Binding Assay:* IL-3 and GM-CSF were radio-iodinated by the iodine monochloride method (Contreras 1983). ^{125}I-IL-5 was purchased from Dupont NEN (North Sydney, NSW, Australia). Binding assays were performed as previously described (Lopez *et al* 1989). Briefly, $1-2 \times 10^6$ TF-1.8 cells were preincubated with BION-1 Fab fragments, anti-βc or control MoAbs over a
15   concentration range of 0.06 to 4200 nM for 1 hour. Radio-labelled ligand was then added and incubated for a further two hours before the cells were separated from free label by spinning through FCS. Counts associated with the resulting cell pellets were determined by counting on a γ counter (Cobra Auto Gamma; Packard Instruments Co, Meridien, CT). Non-specific binding was determined
20   for each ligand by binding in the presence of a 200 fold excess of unlabelled cytokine.

MoAb binding assay: MoAbs were radio-iodinated by the chloramine-T method (McConahey 1980). Saturation binding studies were performed by incubating
25   2×10^6 TF1 cells in a range of concentrations of radio-labelled antibodies in the presence or absence of excess unlabelled antibodies. The binding affinity of each anti-βc MoAb to its antigen was determined by Scatchard transformation (Scatchard 1949) and analysed with the ligand program (Munson and Rodbard, 1980). Competition binding experiments were set up by preincubating the TF1.8
30   cells with a range concentration of IL-3, or GM-CSF, or IL-5 prior to adding radio-labelled MoAb QP1 for two hours as per ligand binding assay. Epitope analysis was determined by testing the capacity of each unlabelled MoAb to compete for the binding of each radio-labelled MoAb to the βc on COS cell transfectant.
35

Co-immunoprecipitation of α and β chains and the βc phosphorylation assays: M07e cells were surface labelled with ^{125}I by the lactoperoxidase method as

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described previously (Walsh and Crumpton, 1977). The labelled cells incubated in ether medium containing IL-3 (100 ng/ml) alone or IL-3 together with the MoAb QP1, 1C1 (0.5 mg/ml) or 7G3 (30 mg/ml) for 5 min. Cells were lysed in lysis buffer consisting of 137 mM NaCl, 10 mM Tris-HCl (pH 7.4), 10%

5   Glycerol, 1 % Nonindet P40 with protease and phosphatase inhibitors (10 mg/ ml lcupeptin, 2mM phenylmethlysulphonyl fluoride, 10 mg/ml aprotonin and 2 mM sodium vanadate) for 30 min at 4°C followed by centrifugation of the lysate at 10,000 x g for 15 min to remove cellular debris. The lysate was precleared with mouse-Ig-coupled Sepharose beads for 18 h at 4°C and incubated with

10   anti IL-3Ra, anti-βc MoAb beads for 2 hr at 4°C. The beads were washed 6 times with lysis buffer and immunoprecipitated proteins were separated by SDS-PAGE under reducing condition. The immunoprecipitated proteins were detected by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The gels were then reprobed by Western blotting analysis with an anti-phosphotyrosine MoAb,

15   3-365-10 (Boehringer Mannheim, Frankfurt, Germany).

TF-1.8 cell proliferation assay: TF-1.8 cells were grown in the presence of 2 ng/ml of GM-CSF. The cells were starved for 24 hours before setting up proliferation assays as described previously (Sun *et al.,* 1996). From

20   dose-response curves the half-maximal proliferation dosage of IL-3 (0.3 ng/ml), GM-CSF (0.03 ng/ml), IL-5 (0.3 ng/ml) or EPO (5 ng/ml) was chosen to perform proliferation experiments in the presence of a range of concentrations of MoAbs. The ^{3}H-Thymidine incorporation of each sample was determined by liquid scintillation and expressed as disintegrations per minute (DPM).

25

Eosinophil survival assays: The maximal dose of IL-5 required to support eosinophil survival after 36 hours was determined. Eosinophils were then cultured with 1 nM of IL 5 plus anti-βc MoAbs for 36 hours. The viability of eosinophils was quantitated by propidium iodide staining and flow cytometry analysis as

30   described (Nicoletti, 1991).

CD69 expression: CD69 expression on eosinophils was measured by means of an anti CD69 monoclonal MoAb coupled to PE by flow cytometry.

35   *βc mutants and MoAb Mapping:* Single amino acid substitutions in the B'-C' and F'- 3' loops of domain 4 of the βc have been described previously (Woodcock, *et*

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al., 1994; 1996). The cDNAs for wild type βc and each of the βc mutants in the B'-C' and F-G' loops were introduced into COS cells by the electroporation (Hercus *et al.*, 1994). Cell transfectants were analysed for surface expression with 48 hours after transfection. Mutants on the B' and C' β-strands such as

5   L356N, W358N, I374N and Y376N were expressed on FDCP1 cells from retroviral expression constructs (Jenkins *et al.*, 1995). Epitope-mapping of anti-βc antibodies was analysed by Immunofluorescent study. The anti-βc MoAbs were tested for their abilities to recognise wild type βc and the βc mutants analysed by flow cytometer using standard immunofluorescence method. For each mutant, the

10   experiment was repeated at least twice.

BION-1 binding inhibitory peptides: E. *coli* derived soluble β_c domain 4 (sβ_c#4) was coupled to Maxisorp ELISA plates at 10µg/ml in 0.1M carbonate buffer overnight and then blocked with 1% BSA. B45.pep (FHWWWQP-

15   GGGCDYDDDK) was derived from four rounds of biopanning the Ph.D-7mer library with sβ_c#4 using an acid eluant. YB12.pep (FPFWYHAHSPWS-GGGCDYKDDDK) was derived from biopanning the Ph.D-12mer library with sβ_c#4 using an acid eluant. B45 was allowed the bond to sβ_c#4 at 0.0125µM and YB12 was allowed to bind sβ_c#4 at 0.025µM. The plate was washed in TBS +

20   0.5% Tween. BION-1 was added to the plates at a starting concentration of 5µg/ml and serial dilutions were used to titrate the BION-1 down to 0.004µg/ml. The plate was washed again and BION-1 binding to sβ_c#4 was detected with α-mo ise conjugated to HRP, using a colour based reaction which was read on a plate counter by absorption.

25

BION-1 inhibition of chronic myelomonocytic cells: Peripheral blood from a patient with chronic myelomonocytic leukemia was centrifuged over Ficoll-Paque to separate the mononuclear cells. After washing and counting, the cells were plated on agar as a concentration of 10^5 per plate. After incubation in medium

30   containing monoclonal antibodies BION-1 or 1C1, with or without IL-3, for 14 days at 37°C the number of arising colonies were counted by mycroscopical examination. Each cell cluster containing more than 40 cells was counted as a colony.

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Results

Development of MoAb BION-1

Previous experiments have shown that the putative F'-G' loop of βc contains a common binding site for IL-5, GM-CSF and IL-3 (Woodcock *et al.* 1996: WO
97/28190). We have now produced a blocking compound, represented by MoAb BION-1, by immunizing mice with COS cells transfected with a cDNA coding for domain 4 of βc. Screening of hybridoma supernatants was performed on a CHO cell line expressing domain 4 of βc. One hybridoma cell line was identified which produced a MoAb which specifically recognized this cell line and not a parental
CHO cell line not expressing domain 4 of βc. This MoAb was termed BION-1 and was characterized in biochemical, binding and biological experiments.

BION-1 recognizes domain 4 as well as wild type βc.

MoAb BION-1 was tested for reactivity against cell lines transfected with βc and
against primary cells known to express IL-5, GM-CSF and IL-3 receptors.
BION-1 recognized COS cells transiently transfected with βc, CHO cells permanently transfected with βc, the erythroleukaemic TF-1 cell line, and purified peripheral blood human neutrophils, eosinophils and monocytes (Figure 1).

The antigen recognized by BION-1 was confirmed to be domain 4 of βc, by
biochemical analysis of transfected cells. Figure 2A shows that BION-1 immunoprecipitated a surface ^{125}I-labelled protein of about 120,000 MW consistent with the size of βc. Similarly, BION-1 recognized a protein of 120,000 MW by Western blotting using lysates of CHO cells expressing full length wild type βc
(Figure 2B). The size of these bands also corresponded to the bands recognized by a previously developed anti-βc MoAb (Korpelainen *et al* 1993; Woodcock *et al.* 1996). To formally show that BION-1 recognized domain 4 of βc we also tested BION-1 for its ability to immunoprecipitate domain 4 expressed on the surface of CHO cells. As a positive control we incorporated a short polypeptide to the N-
terminus of domain 4 (flag epitope) to which a MoAb has been previously developed. As a negative control, we used the anti-βc MoAb 1C1 which recognizes an epitope located elsewhere in βc. Figure 2C shows that BION-1 immunoprecipitated a band of about 80,000 MW from ^{125}I-surface labelled-domain 4-expressing CHO cells consistent with the expected size of domain 4. MoAb M2
against the flag epitope added to domain 4 of βc also precipitated a similar size protein. In contrast, MoAb 1C1 failed to immunoprecipitate domain 4. These

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experiments show that BION-1 can specifically recognize domain 4 of βc on the surface of cells and following denaturation of the protein.

BION-1 inhibits the high affinity binding of IL-5, GM-CSF and IL-3 to TF-1 cells

5   and to human eosinophils

Given that domain 4 of βc is crucial for the high affinity binding of IL-5, GM-CSF and IL3, we examined whether BION-1 was able to affect this binding. We found tha t BION-1 inhibited in a dose-dependent manner the binding of ^{125}I-IL-5, ^{125}I-GM-CSF and ^{125}I-IL-3 to the human erythroleukaemic cell line TF-1. For each

10   radioligand we used the smallest possible concentration to maximize the possibility of measuring high affinity. This can be more readily achieved with IL-3 and GM-CSF for which the difference between the low affinity component (provided by each α chain alone) and the high affinity component (provided by co-expressing βc with each α chain) is about 1,000 fold and 30 fold respectively.

15   In the case of IL-5, the affinity conversion of βc is only in the 25 fold range, hence, high and low affinity binding cannot be clearly separated. This is likely to explain why BION-1 shows complete inhibition of ^{125}I-GM-CSF and ^{125}I-IL-3 binding (Figure 3). The residual ^{125}I-IL-5 binding seen with high concentrations of BION-1 is likely to be the result of low affinity ^{125}I-IL-5, binding (α chain)

20   which BION-1 would not be expected to inhibit. This is consistent with BION-1 inhibition of ^{125}I-IL-5, binding reaching a plateau beyond which no further inhibition can be detected (Fig 3a). Other anti-βc MoAb (anti-.βc control) and the IgG$_1$ MoAb control did not inhibit ^{125}I-IL-5, ^{125}I-GM-CSF and ^{125}I-IL-3 binding to TF-1 cells (Figure 3).

25

The blocking effect of BION-1 was seen whether the MoAb was used as purified IgG or as Fab' fragment. Figure 4 shows that the Fab' fragment of BION-1 blocked the binding of 50 pM ^{125}I-IL-5, 50 pM ^{125}I-GM-CSF and 200 pM ^{125}I-IL-3 to TF-1 cells.

30

Since one of the major clinical utilities of blocking IL-5, GM-CSF and IL-3 binding is likely to be in asthma, a disease in which eosinophils are believed to play a major role, it was important to test whether BION-1 could block the binding of IL-5, GM-CSF and IL-3 to these cells. As shown in Figure 5, BION-1

35   inhibited the binding of all three radio-labelled cytokines to purified human

20

eosinophils. In contrast, other anti-βc MoAb or the IgG₁ MoAb control failed to
do so.

Epitope mapping of BION-1

5　The fact that BION-1 inhibited the binding of ^{125}I-IL-5, ^{125}I-GM-CSF and ^{125}I-IL-3 to TF-1 cells and eosinophils suggested that it might be binding to the critical region in βc to which these cytokines bind or at least in close proximity to it. To try to define the region/epitope in βc recognized by BION-1, we used several mutants of βc and examined whether substitutions of individual amino acids in the

10　predicted B'-C' loop or F-G' loop impaired BION-1 binding. Two sets of experiments were carried out. In the first instance we immunoprecipitated wild type βc from transfected COS cells with a MoAb anti-βc. The immunoprecipitates were then tested for reactivity with the control anti-βc MoAb IC1, or BION-1. The results shows that βc mutants carrying the substitutions M363A/R364A, or

15　E366A or R418A were not recognized by BION-1 (Figure 6). In a second set of experiments, the direct binding of radio-labelled BION-1 was measured on transfectants expressing the same mutants. Similar results were obtained in that whilst IC1 bound with similar affinity to wild type βc and the βc mutants, BION-1 binding was eliminated by the M363A/R364A, E366A and R418A mutants (Table

20　I). These results suggest that the epitope recognized by BION-1 is formed, at least in part, by M363 and/or R364, E366 and R418. This is consistent with the disclosure in WO 97/28190 that agents that bind the putative F-G' loop (of which R418 is part of) will be antagonists of IL-5, GM-CSF and IL-3.

25　### BION-1 and IL-3 reciprocally inhibit each other's binding
To confirm that the epitope recognized by BION-1 was the same or close to the binding site utilized by IL-5, GM-CSF and IL-3, we performed the reverse experiment, in which BION-1 was radio-labelled and increasing concentrations of IL-3 used to compete for ^{125}I-BION-1 binding. The results showed (Figure 7) that

30　IL-3 competed for ^{125}I-BION-1 binding in a dose-dependent manner emphasizing the close and intimate proximity of BION-1 and IL-3 binding epitopes in βc

BION-1 specifically inhibits the function of IL-5, GM-CSF and IL-3 including their stimulation of eosinophil production and activation.

35　To ascertain whether the inhibition of IL-5, GM-CSF and IL-3 binding by BION-1 was translated into inhibition of IL-5, GM-CSF and IL-3 stimulation we used the factor dependent TF-1 cell line. This cell line proliferates in the presence

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of either IL-5, GM-CSF, IL-3 or erythropoietin (EPO) (Figure 8). As shown in
Figure 8 MoAb BION-1 but not other MoAb anti-βc nor an IgG_1 control MoAb
inhibited the stimulation of TF-1 cell proliferation by IL-5, GM-CSF and IL-3. In
contrast, the stimulating ability of erythropoietin was not inhibited showing

5   specificity of BION-1 for the IL-5/GM- CSF/IL-3 receptors system.

Titration experiments showed that BION-1 inhibited cytokine-mediated TF-1 cell
proliferation in a dose-dependent manner with an ED_{50} of about 100-300 nM
(Figure 9). Figure 9 also shows that other anti-βc MoAb were not inhibitory, and

10   that Fab fragments of BION-1 behaved similarly to BION-1 as a whole IgG with
virtually overlapping ED_{50} values.

Since eosinophils are believed to be the major effector cells in asthma and they
respond to IL-5, GM-CSF and IL-3, we examined BION-1 for its ability to block
15   eosinophil production, eosinophil survival and eosinophil activation in response to
these three cytokines. We found that BION-1 but not MoAb 8E4 inhibited the
ability of IL-5, GM-CSF and IL-3 to stimulate the formation of eosinophil
colonies from human bone marrow cells (Table II).

20   Importantly, BION-1 inhibited the pro survival activity of IL-5, IL-3 and GM-CSF
on purified peripheral blood human eosinophils. Whilst these cytokines are
essential for maintaining eosinophil viability (Figure 10A), blocking of βc by
MoAb BION-1 promotes eosinophil cell death to levels similar to those observed
in the absence of cytokines (Figure 10B). Eosinophils can be activated by IL-5,
25   GM-CSF and IL-3 as well as by tumour necrosis factor (TNF-α), a factor that
operates through the TNF-α receptor. A sign of eosinophil activation is the
upregulation of the CD69 surface antigen, a phenomenon induced by all four
cytokines (Figure 11A). Using this activation system we found that BION-1
inhibited the activation of eosinophils by IL-5, GM-CSF and IL-3 (Figure 11B).
30   Other MoAb anti-βc or IgG_1 controls failed to do so. In addition the blocking effect
of BION-1 was found to be specific in that the stimulating activity of TNF-α was
not inhibited (Figure 11B).

BION-1 specifically inhibits IL-3 receptor dimerization and activation
35   In order to define the mechanism of BION-1 antagonism we examined BION-1 for
its ability to influence receptor dimerization and activation. We have previously
shown that IL-3 or GM-CSF or IL-5 induce dimerization of the respective α

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cha ns with βc. a phenomenon that leads to receptor activation as measured by
tyr sine phosphorylation of βc. This is confirmed here, with Figure 12 showing
tha: in the absence of cytokines antibodies to the α chain (left panel), or βc (right
par 2l). immunoprecipitate their appropriate antigens (α chain and βc respectively).

5   In t ie presence of IL-3, dimerization of α and βc takes place allowing either anti-α
chain or anti βc MoAb to immunoprecipitate both receptor subunits. This is
acc:mpanied by tyrosine phosphorylation of βc (top panel). We show in this
figl.re that pre-incubation of the cells with BION-1 blocks receptor dimerization
anc tyrosine phosphorylation of βc. As a control we used the anti β_c MoAb 1C1

10   wh ch was unable to prevent receptor dimerization and activation.

BI( )N-1 specifically inhibits chronic myelomonocytic cell growth
BI( )N-1 is shown to inhibit the activity of one or all of IL-5, IL-3 & GM-CSF
me liated effectors of leukaemic cells. In particualr BION-1 inhibits growth in

15   vit o of chronic myelomonocytic cells (CMML), whereas a control antibody (1C1)
do s not (Figure 14). Furthermore, BION-1 inhbits even in the presence of IL-3
wh reas the control does not.

Sci eening and isolation of new inhibitory compounds
20   A l irge range of potential therapeutic compounds that might act as antagonists, or
pei haps agonists of IL-3, GM-CSF and IL-5 individually or collectively, can be
rea lily screened. The screening is initially to determine whether the binding of
BI)N-1 or a fragment thereof to βc receptor or fragment is inhibited. The nature
of .hese inhibitory compounds will not be limited, and the methods used for a

25   bir ding assay can be any one of the many techniques known to those skilled in the
art Such methods may include affinity selection chromatography, ultrafiltration
ass ays, the scintillation proximity assay, interfacial optical techniques, the quartz
cr) stal microbalance, the jet ring cell, interferometric assays using porous silicon
to mmobilise the receptor. Reference to such techniques can be found in

30   W· iodbury et al 1999, which reference is incorporated herein in its entirety.

Th range of therapeutic compounds may include peptides, oligonucleotides, or
otl.er small organic or inorganic molecules. Figure 13 shows the results of
sc: eening 7-mer and 12-mer peptide libraries using soluble β_c domain 4 supported

35   on ELISA plates.

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DEPOSIT OF CELL LINE

The cell line BION-1 was deposited on the April 29th, 1998 in the American Type
Culture Collection (ATCC) at 101801 University Boulevard, Manassas, Virginia,
United States of America and has been designated ATCC HB-12525.

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Table I   Epitope mapping of Bion-1. Binding affinities of MoAb Bion-1
               tested on COS cells transfected with wild type βc or mutants of βc

βc wild type:	Bion-1 KD	ICl KD
	49.3*	4.4
βc mutated in the B'-C' loop:		
M363A/R364A	0 †3.8	
Y365A	69.41.5	
E356A	02.4	
H367A	27.02.8	
1358A	21.72.4	
D369A/H370A	32.43.6	
βc mutated in the F'-G' loop:		
R418A	0	1.8
T419A	23.3	3.2
G420A	53.0	1.5
Y421A	38.9	2.3

* K_D in nM

0† = not detectable binding

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Table II   Inhibition of IL-5, GM-CSF and IL-3 mediated eosinophil colony formation by BION-1

	Medium	[MoAb 8E4] (100 µM)	[MoAb BION-1] (µM)			
			0.1	1	10	100
IL-5 (1nM)	13 ± 4*	15 ± 3	13 ± 4	8 ± 2	2 ± 2	2 ± 0
GM-CSF (2nM)	9 ± 4	18 ± 4	20 ± 4	13 ± 4	2 ± 2	0 ± 0
IL-3 (2nM)	4 ± 2	8 ± 1	8 ± 2	4 ± 1	1 ± 1	0 ± 1
NONE	0 ± 0	0 ± 0	0 ± 0	0 ± 0	2 ± 0	0 ± 0

*Number of day 14 eosinophil colonies per 10^5 seeded bone marrow cells. Values shown are the mean from triplicate determination ± SEM.

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WO 00/095(1   PCT/AU99/00659

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